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Methods in Molecular Biology

Ribozymes

Chapter 15

Single Molecule FRET Characterization of Large Ribozyme Folding

Lucia Cardo[a], Krishanthi S. Karunatilaka[b], David Rueda[b,*] and Roland K. O. Sigel[a,*]

[a] *Institute of Inorganic Chemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland; *email, roland.sigel@aci.uzh.ch*

*b Wayne State University, 5101 Cass Ave., Detroit 48202 Michigan, USA; *email, david.rueda@wayne.edu*

Running Head: RNA folding by smFRET

Abstract

A procedure to investigate the folding of group II intron by *single molecule Fluorescence Resonance Energy Transfer* (smFRET) using total internal reflection fluorescence microscopy (TIRFM) is described in this chapter. Using our previous studies on the folding and dynamics of a large ribozyme in the presence of metal ions (i.e. Mg^{2+} and Ca^{2+}) and/or the DEAD box protein Mss116 as an example, we here describe step-by-step procedures to perform experiments. smFRET allows the investigation of individual molecules, thus, providing kinetic and mechanistic information hidden in ensemble averaged experiments.

Key Words: single molecule, FRET, group II introns, folding, TIRF microscopy, dwell times, DEAD box helicases.

1. Introduction

In the last two decades, bio and nanotechnologies have focused on the development of methods capable of analyzing the structure/function correlation of biomolecules at the single molecule level (**1-7**). Such methods aim to overcome some of the limitations of bulk experiments. Ensemble averaged experiments yield an average dynamic behavior from a large number of molecules, thus, any unsynchronized, dynamic information about short lived or low populated intermediates can often be lost or misinterpreted. Instead, observing the behavior of single molecules in real time allows the direct analysis of individual folding pathways of different molecules within a large ensemble, including short-lived intermediates, without need for synchronization. Thus, the distribution of different behaviors, rather than the average of these distributions, originating from the contribution of each single observed molecule, can be determined by single molecule experiments.

Ribonucleic acids (RNAs) are highly versatile systems with a large conformational diversity and intricate folding pathways, which are regulated by metal ions and often by other molecules (mainly proteins) *in vivo* (**8-11**). The analysis with 'traditional' methods, such as NMR, CD, UV melting or electrophoresis techniques, provides essential information regarding RNA folding (**12-16**), but studies of single molecules of RNA performed in the last years have helped to uncover new insights that bulk experiments could not reveal(**7**).

Single molecule fluorescence resonance energy transfer (smFRET) (**17-26**) is one of the most widely used methods to observe the folding of single RNAs (**7,17,27-32**). In a standard FRET experiment, the biomolecule of interest is labeled with a donor-acceptor fluorophore pair. Upon excitation of the donor, the efficiency of the energy transfer from the donor to the acceptor is dependent on the distance between the two fluorophores (*see* **Note 1**) (**1**). Thus, FRET can be used as a molecular ruler in the 2-10 nm distance range, making it an ideal tool to study the structural dynamics and function of biomolecules.

The interference of background signals from various fluorescence sources is one of the main issues in detecting single fluorophores. This can be minimized by measuring highly diluted samples (pM) and using methods that permit the excitation of very small sample volumes (μm^3). Two strategies are commonly employed: confocal microscopy and total internal reflection fluorescence microscopy (TIRFM) (*see* **Note 2**) (**33,34**). Here, we focus on the application of TIRFM to detect FRET of single molecules of RNA, using the example of the Sc.ai5γ group II intron ribozyme from *Saccharomyces cerevisiae*. The folding pathway of this large RNA was characterized by smFRET revealing a new folding paradigm for this large RNA and showing that increasing amounts of Mg^{2+} not only fold the RNA but also increase the dynamic behavior of the single domains (**35**). The addition of small amounts of Ca^{2+} instead leads to the formation of two distinct subpopulations (**36**) whereas the DEAD-box protein Mss116 can substitute for a large part of Mg^{2+} and stabilizes the active state (**37**).

Group II introns belong to the class of large phosphoryltransfer ribozymes together with group I introns and RNase P RNA. Although the sequence conservation in group II introns is very low, their secondary structure is greatly conserved, usually divided into six subunits, identified as domains D1-D6 (**38**). D1 is the largest domain and provides the scaffold for the docking of other domains, whilst D5 is the most conserved domain and comprises a large part of the catalytic core. The natural *S. Cerevisiae (Sc.)* ai5γ group IIB intron (yeast mitochondrial intron residing in the *cox1* gene, ~900 nb) is one of the best characterized introns of this category (**38**). Its folding (and consequently its activity) requires high ionic strength *in vitro*, but splicing *in vivo* is also assisted by protein cofactors (**39-41**). The folding dynamics of this intron has been investigated by smFRET by annealing two short DNA strands (15-20 nts) each functionalized with either Cy3 or Cy5 (**17**) (*see* **Note 3**). The synthetic Cy3-DNA and Cy5-DNA oligonucleotides were annealed to a modified version of *Sc*.ai5γ group II intron named *Sc*.D135-L14 (**Fig. 1**) that includes D1, D3 and D5 as well as two 15 nts loops within D1 and D4 whose sequences are complementary to the DNA oligos (*see* **Note 4**). Additionally, the 3'-end of the intron is elongated with a sequence complementary to a third DNA strand functionalized with biotin at its 5'-end. Such, immobilization of the RNA can be achieved on the surface of a streptavidin coated quartz slide via the strong streptavidin-biotin interaction.

Before advancing to the smFRET experiments, the correct (and optimal) annealing of Cy3-, Cy5- and biotin-DNAs must be confirmed by native gel electrophoresis. Furthermore, all parameters influencing R_0 (and consequently also FRET efficiency) are constants (*see* **Note 1**) known for every pair of dyes, except κ^2 that is the factor related to the reciprocal orientation of fluorophores dipoles in the space. The fluorophores are usually assumed to freely rotate without conformational constrictions, meaning that an average value of $\kappa^2=2/3$ can be applied in calculating FRET efficiency. Fluorescence anisotropy measurements

(**18,42**) can be performed to confirm that donor and acceptor are indeed rotating freely, which is the case for *Sc.*D135-L14 (**35**).

The immobilized and fluorophore-carrying RNA/DNA complex is analyzed on a slide containing a home-built microfluidic chamber placed on an inverted microscope (**Fig. 2a,b**) (**17,33**). To achieve total internal reflection and visualize the single molecules, the laser excitation beam reaches the slide through a quartz prism placed over the slide itself (**Fig. 2b**). The beam is totally reflected without penetrating below into the sample if the incidence angle is larger than the critical angle (θ_c) (*see* **Note 5**). Such, an evanescent wave is created which diffuses only shortly (100-200 nm) below the quartz/solution interface and only the molecules present in that small volume are excited (*see* **Note 6**). The fluorescence emission from the sample is collected through an objective and directed into a light-tight box containing a set of dichroic mirrors and lenses that separate the donor and acceptor wavelengths(**33**). The two signals are then simultaneously detected with a CCD camera as two individual images. The acquisition software allows observation of both the donor and acceptor channel in real time displaying the single molecules as tiny bright dots (**Fig. 2c**). Each pair of dots (from the donor and acceptor channels) is analyzed with the home-built software to determine the corresponding time trajectories of their smFRET efficiencies. The apparent FRET efficiency (**43**) is calculated as:

$$\mathrm{FRET} = \frac{I_A}{I_A + I_D}$$

where I_A and I_D are the emission intensities of acceptor and donor, respectively, as integrated from each pair of dots. The image of immobilized fluorescent microspheres (beads) is used as calibration tool to map the two channels. This control is recommended in order to achieve a perfect correlation between two signals in the two channels relative to the same single molecule (**33**).

In the following we first describe the preparation of *Sc.*D135-L14 ribozyme by *in vitro*

transcription, including the native gel and fluorescent anisotropy control experiments. The smFRET section then includes the description of slide preparation, the execution of smFRET experiment and the data analysis. As an example, the smFRET experiments and analysis of the Sc.ai5γ-L14 group II intron construct in dependence of $MgCl_2$ concentration or in the presence of the DEAD box protein Mss116 are described.

2. Materials

All chemicals used for preparing buffers and stock solutions are at least puriss p.a. and purchased from usual suppliers. Buffers and solutions are prepared using double distilled autoclaved H_2O (ddH_2O) and subsequently filtered using 0.2 μm sterile filters (Filtropur S syringe filters for volumes up to 100 mL or Steritops Express^{TM}PLUS bottle top filters for larger volumes). Polyacrylamide gels are prepared using AccuGel^{TM} 29:1 (acrylamide:bisacrylamide; 40% w/v) stabilized solution from National Diagnostic (UK). Glassware and consumables (eppendorfs, falcon tubes, pipette tips, etc) must be either autoclaved or bought as sterile and DNase/RNase free items.

2.1. General Stock solutions

The following stock solutions should be at hand in order to prepare the buffers and solutions listed below:

1 M Tris·HCl, pH 7.5 (2-Amino-2-hydroxymethyl-propane-1,3-diol·HCl, pH 7.5); 1 M EDTA, pH 8.0 (ethylenediamine-N,N,N',N'-tetraacetic acid); 5 M NaOH; 500 mM HEPES, pH 7.5 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); 100 mM MOPS, pH 6.0 (3-(N-morpholino)propanesulfonic acid); 5 M NaCl.

2.2. Buffers and Solutions for RNA Sc.D135-L14 Transcription

1. *Stock solutions of nucleoside 5'-triphosphates (NTPs).* Adenosine 5'-triphosphate (ATP; GE Healthcare), guanosine 5'-triphosphate (GTP; GE Healthcare), cytidine 5'-triphosphate (CTP; GE Healthcare), uridine 5'-triphosphate (UTP; Acros-Brunschwig). About 130 mg of NTP are dissolved in 800 μL of H_2O and 20 μL of 1 M Tris·HCl each. The pH is adjusted with freshly prepared 5 M NaOH to pH 7.0 and the solution filled up to 1 mL with ddH_2O. The exact concentration of the freshly prepared NTP solutions is determined by UV spectroscopy (*see* **Note 7**). Aliquots of ~200 μL each are then stored at –20 °C and used for transcription within a few months.

2. *pT7D135-L14 plasmid stock solution.* This plasmid encodes the D135-L14 sequence and was stored at a concentration of ~0.3 mg/mL in ddH_2O at –20 °C. Standard digestion with *Hind*III (or any other specific restriction enzyme) followed by phenol-chloroform extraction is done before *in vitro* transcription with T7 RNA polymerase. The concentration of the cut plasmid is determined based on its absorbance at 260 nm (double-stranded DNA average extinction coefficient $\varepsilon = 0.020$ μg $mL^{-1}cm^{-1}$.

3. *10x Transcription buffer (pH 7.5), 5 mL.* 400 mM Tris·HCl (pH 7.5), 400 mM DTT (1,4-Dithio-DL-threitol), 100 mM spermidine, 200 mM $MgCl_2$, 0.1% Triton X-100.

4. *T7 RNA polymerase*. Homemade T7 RNA polymerase (**44,45**) or commercially available T7 polymerase can be used.

5. 100 mL cold ethanol (–20°C) and 10 mL 5 M NaCl

2.3. Buffers and Solutions for Polyacrylamide Gel Electrophoresis (PAGE).

1. *Denaturing electrophoresis buffer*: 1x TBE buffer (~700 mL for preparative gel) prepared from 10x TBE buffer (Tris-borate-EDTA; 0.89 M Tris, 0.89 M boric acid pH 8.3, 20 mM Na_2EDTA; from National Diagnostic, UK).

2. *Denaturing gel loading buffer, 10 mL.* 8 M Urea, 2 mM Tris (pH 7.5), 20 mM EDTA,

0.02% xylene cyanol, 0.02% blue bromophenol (store at 4 °C).

3. *Denaturing 5% PAGE gel solution, 500 mL.* 12.5 mL of AccuGel™ 29:1 (acrylamide:bisacrylamide; 40% w/v), 21 g Urea (ultrapure grad, from EUROBIO, France) and 10 mL 10x TBE (Tris-borate- EDTA) buffer (from National Diagnostic, UK) are diluted with ddH_2O to 100 mL. Gel solutions are best prepared one day prior to use, filtered after preparation, and stored in the dark at 4°C. For a 18% gel use 45 mL AccuGel™.

4. *Native gel electrophoresis buffer, 5x stock, 300 mL, pH 7.4.* 330 mM HEPES, 170 mM Tris·HCl (pH 7.5), 15 mM magnesium acetate. The pH of the solution is adjusted to 7.4 by adding 5 M NaOH. Electrophoresis buffer is used at 1x.

5. *Native gel loading buffer, 1 mL* 60% glycerol.

6. *Native 6% PAGE gel solution, 100 mL.* 16 mL of AccuGel™ 29:1 (40% w/v), 20 mL 5x native gel buffer diluted with ddH_2O to 100 mL total.

7. *Gel casting solutions:* 500 μL of 10% Ammonium persulfate and 50 μL TEMED (tetramethylethylenediamine) for each 100 mL of gel casting solution (either denaturing or native gel).

8. *Elution buffer (pH 6.0), 50 mL*: 10 mM MOPS, 1 mM EDTA, 250 mM NaCl. The buffer should be kept at 4°C and protected in alumina foil after preparation.

2.4. Buffers and Solutions for smFRET experiments.

1. *Cy3 and Cy5 labeled DNA and T-Biotin-DNA oligonucleotides* are purchased from Microsynth, Balgach (Switzerland) or from HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven CT (USA), purified by gel 18% denaturing polyacrylamide gel electrophoresis, re-dissolved in 200 μL of ddH_2O and stored in the dark at –20°C. Concentrations are measured by UV-Vis, using the "nearest neighbour" based method for T-Biotin-DNA (*see* **Note 8**), and the following molar extinction

coefficients for the fluorophore-DNA oligos: $\varepsilon_{Cy3\text{-}DNA}$ (550 nm) 150'000 $M^{-1}cm^{-1}$, $\varepsilon_{Cy5\text{-}DNA}$ (647 nm) 250'000 M^{-1} cm^{-1}.

2. *Catalase* from bovine liver (crystalline suspension in water containing 0.1% thymol, by Sigma) and *Glucose Oxidase* Type VII (Sigma-Aldrich).

3. 0.5 mL of *1 mg/mL Biotinylated BSA solution* (ImmunoPure Biotinylated Bovine Serum Albumine by Fisher Thermo Scientific) and 0.5 mL of *0.2 mg/mL Streptavidin solution* (from *S.avidinii*, by Invitrogen). Both solutions are stored at 4°C.

4. *Fluorescent beads*. FluoroSpheres® carboxylate-modified microspheres (0.2 μm, red fluorescent, 2% in distilled water, 2 mM azide) from Invitrogen are used. Keep at 4°C and protected from light (*see* **Note 9**).

5. *5x Reaction buffer (pH 6.9), 10 mL*: 400 mM MOPS, 2.5 M KCl. Keep at 4°C and cover with aluminum foil.

6. *T50 buffer (pH 7.5), 1 mL:* 50 mM Tris·HCl, 50 mM NaCl.

If also proteins are present in the reaction mixture, i.e. in the described case Mss116, the following additional buffers and solutions have to be prepared

7. *Solutions for preparation of PEG-coated slides:* 3-aminopropyltriethoxysilane (Vectabond reagent, Vector Laboratories, Inc., Burlingame, CA), biotin polyethylene glycol succinimidyl carboxymethyl (BIO-PEG-SCM, 3400/5000 MW, Laysan Bio. Inc., Arab, AL) and methoxy polyethylene glycol succinimidyl carboxymethyl (m-PEG-SCM, 5000 MW, Laysan Bio. Inc., Arab, AL).

8. *5x Reaction buffer for protein experiments (pH 7.5), 5 mL:* 200 mM MOPS, 500 mM KCl. Keep at 4°C and cover with aluminum foil.

9. *1x Reaction buffer for protein experiments (pH 7.5), 5 mL:* 40 mM MOPS, 100 mM KCl and 8 mM $MgCl_2$. Filter to sterilize and keep at 4°C covered with aluminum foil.

10. *10% Sugar-buffer for protein experiments (pH 7.5), 5mL:* 10% D-glucose (w/v), 40 mM

MOPS, 100 mM KCl and 8 mM $MgCl_2$. Filter to sterilize and keep at 4°C covered with alumina foil.

11. *100 mM ATP (pH 7), 1 mL.*

3. Methods

3.1. RNA Transcription and Purification

For a more detailed description see also Section 3.1 of Chapter 16 *Metal Ion-RNA Interactions Studied via Multinuclear NMR*.

1. 5 mL *in vitro* transcription of *Sc.*D135-L14: Mix cut pT7D135-L14 plasmid (7.5μg/mL final concentration in 5 mL) with 1x transcription buffer, ATP, CTP, GTP and UTP (final concentration of 5 mM in 5 mL for each NTP) and add ddH_2O up to 4.9 mL. Aliquot the reaction solution into five different eppendorf tubes (980 μL in each tube) and add 20 μL of T7 RNA polymerase to each tube (*see* **Note 10**). Shake the reaction mixtures for 4-5 hours at 37 °C and 300 rpm (overnight is also possible for shorter RNAs).

2. Spin down the insoluble magnesium pyrophosphate resulting from the transcription (1'500 g for 5 min), combine the supernatants into a falcon tube, add 5 M NaCl (final concentration of 250 mM NaCl), and mix with cold ethanol (3x volume). Store at –20°C (at least 6 hours, better overnight) or, alternatively, at –80°C for 1 hour .

3. Centrifuge at 4 °C and 13'000 g for 40 min. Separate the white pellets from the supernatant by decantation and dissolve the pellets in as little of ddH_2O as possible (typically ~2 mL for a 5 mL transcription). Keep the solution on ice.

4. Add an equal volume of denaturing loading buffer and purify by denaturing 5% polyacrylamide gel electrophoresis (PAGE). Usually the product solution of a 5 mL *in vitro* transcription is split onto two preparative gels (28x42 cm with 1.5 mm spacers).

5. The RNA bands are located by UV-shadowing, excised and extracted from the gel by the

crush-and-soak method: Crush the gel into a fine slurry by smashing it through a 10 mL syringe that has been melted at the tip and puckered again with a needle. Transfer the gel into falcon tubes and soak and shake it in Elution Buffer (3x the volume of the gel) for 4 h at 4°C.

6. Separate the eluted RNA from the gel by centrifugation and precipitate with 3 volumes of cold ethanol. Centrifuge at 4 °C and 13'000 g for 40 min.

7. After separation from the supernatant, the white pellets are vacuum-dried in a concentrator ("speed-vac"), re-dissolved in as little ddH_2O as possible and stored at –20 °C.

8. Measure the absorbance at 260 nm and use the following equation valid for large RNAs: conc = A/(#NTP/100), with #NTP = 637 for the D135-L14 RNA construct.

3.2. Control experiments.

3.2.1. Native Gel Electrophoresis

Native gel electrophoresis experiments are performed to verify the correct annealing of the Cy3- and Cy5-DNAs to the RNA under the conditions used in the smFRET experiments.

1. Using the oligonucleotide stock solutions (Section 2.3.), prepare seven samples of 9 μL each with 1x Reaction buffer containing (i) *Sc.*D135-L14 (2 μM), (ii) Cy3-DNA (15 μM) and (iii) Cy5-DNA (15 μM), with the following combinations: (1) only (i), (2) only (ii), (3) only (iii), (4) (ii)+(iii), (5) (i)+(ii), (6) (i)+(iii), (7) (i)+(ii)+(iii). Concentrations given above are the final concentrations. Use dark (brown) eppendorf tubes for samples preparation.

2. Heat to 90 °C for 45 seconds.

3. Add 1 μL of 1 M $MgCl_2$ (100 mM final concentration) right after heating to 90 °C and incubate at 42 °C for 10-15 min.

4. Add 8 μL of loading native buffer to each sample and perform electrophoresis using the 6% native gel (17x24 cm) at 4 °C for 2 hours (15 Watts). Use a molecular imaging scanner to visualize the bands containing Cy3 and Cy5 (we use a Typhoon molecular scanner) (*see* **Note**

11).

5. Additionally, the gel is stained in a GelRed^{TM} bath (5 µL of GelRed^{TM}, Biotium Inc. Hayward CA, in 100 mL ddH_2O) for 15 min followed by 5 min in ddH_2O and detected by UV shadowing at 260 nm to visualise possible bands not containing Cy3 and Cy5.

3.2.2. Fluorescence Anisotropy measurements.

Fluorescence anisotropy measures the rotational diffusion of fluorescent molecules. Linking a fluorophore to a large biomolecule may affect its ability to rotate freely, thus affecting the values of κ^2 and R_0. We performed fluorescence anisotropy measurements (**18,42**) to confirm that Cy3 and Cy5 are freely rotating when bound to the DNA annealed to *Sc.*D135-L14 (**35**) and that $\kappa^2 = 2/3$ can be assumed (*see* **Note 1**).

Prepare four samples containing Cy3-DNA (0.15 µM) and/or Cy5-DNA (0.5 µM, total volume of 200 µL each) in the presence and absence of *Sc.*D135-L14 (0.3 µM) and fold in 100 mM $MgCl_2$ as described above (Section 3.2.1.).

1. Anisotropy experiments are conducted in a cuvette (100 µL) to measure fluorescence polarization using polarized filters on the spectrophotometer. Fluorescence intensities (*I*) of polarized excitation and emission in vertical (v, 0°) and horizontal (h, 90°) positions are recorded in all four possible combinations I_{vv}, I_{vh}, I_{hv}, I_{hh}. The anisotropy value *r* is calculated as described (**42**):

$$r = \frac{I_{vv} - gI_{vh}}{I_{vv} + 2gI_{vh}} \qquad \text{with} \qquad g = \frac{I_{hv}}{I_{hh}} \tag{3}$$

If the observed anisotropy values for the free fluorophores are similar to those of the fluorophores attached to DNA in the *Sc.*D135-L14 RNA-DNA complex is an indication of the free rotation ability of fluorophores in a complex. It is noteworthy that, due their short

fluorescent lifetimes, the fluorescent anisotropies of Cy3 and Cy5 alone in solution are high compared to other commonly used fluorophores such as fluoresceine or rhodamine.

3.3. smFRET Experiments

3.3.1. Instruments

The folding of D135-L14 ribozyme under optimal *in vitro* splicing conditions (500 mM KCl, 80 mM MOPS pH 6.9 and 100 mM $MgCl_2$) or under near-physiological conditions (100 mM KCl, 40 mM MOPS pH 7.5 and 8 mM $MgCl_2$) was monitored using a prism-based total internal reflection fluorescence (TIRF) inverted microscope (IX-71, Olympus, Center Valley, PA). Such a setup has recently been excellently described in detail (**33**) and is thus only shortly summarized here (**Fig. 2**).

In order to obtain total reflection of the laser beam (532 nm, 3 mW, CrystaLaser GCL-532-L, Reno, NV), the angle of incidence (θ) on the slide should be larger than the critical angle (θ_c). When $\theta>\theta_c$, the laser beam totally reflects and creates an evanescent wave at the slide-solution interface that can penetrate a few hundred nanometers to excite the fluorophore-labeled samples (**Fig. 2b**). The laser beam is introduced through a quartz Pellin-Broca prism (CVI Melles-Griot, Albuquerque, NM) by a mirror (Newport, Irvine, CA). The angle of incidence can be controlled by adjusting the height of the mirror. The reflected laser beam is focused to the prism using a BK7 lens with a 100 mm focal length (Newport, Irvine, CA).

The donor and acceptor emissions are collected through an inverted microscopic objective and transferred into a light-sealed box through a slit (**Fig. 2b**). The first dichroic mirror (DM 1, 635DCXR, Chroma, Rockingham, VT) physically separates the donor and acceptor intensities and allows them to pass through lenses (L1 and L2, 200 mm focal length and 2.0 inch diameter) that amplify the image. The second dichroic mirror (DM 2) recombines the separated donor and acceptor emission signals as side-by-side images onto a high quantum

yield CCD camera (Ixon+, DV-897E, Andor, South Windsor, CT). The CCD camera amplifies the signals by the highest electron multiplication (EM) gain to maximize the signal-to-noise ratio and transfers the digitalized frames to a computer for data analysis (**Fig. 2c**).

3.3.2. Preparation of Microscope Slides with a Microfluidic Chamber

Standard microscope quartz slide (76x25x1 mm, Finkenbeiner Inc., Waltham, MA, USA) must be modified to create a microfluidic chamber, in which the sample is loaded and immobilized. The slides must be thoroughly cleaned and protected from dust and any type of contamination. A very detailed description has recently been published (**33**) and is repeated here in slightly different words.

1. Drill two holes into the quartz slides as shown in **Fig. 3**. Use a hand drill (Dremel 300-N, Racine, WI) held by a work-station (Dremel 220-01, Racine, WI) and diamond drill bits (1.0 mm diameter, Kingsley North, Norway, MI). With a marker, spot the positions of the holes. Drill through the slide placed over a sustaining cylindrical ring immersed in a basin filled with enough water to submerge the slide. The diamond drill bit must penetrate the slide very slowly to avoid slide rupture. Use each diamond bit for 5-6 times only.
2. Clean the slides with a thick paste of powder detergent (Alconox, VWR) and water. Rub slides thoroughly with fingers for at least 20 seconds. Rinse with water keeping wiping with fingers to make sure that all the detergent is removed. Rub and rinse with ethanol and again with ddH_2O. The slides should look perfectly clean at the end of this procedure
3. Place the slides in a beaker containing 100 mL of autoclaved water, 20 mL of 30% ammonium hydroxide and 20 mL of 30% Hydrogen Peroxide (work under fume hood). Boil the solution for 20 min, gently stir with a magnetic bar that does not hit the slides and make sure that the whole surface of the slides is submerged in the solution.
4. Use tweezers to take the slides out of the solution, rinse each of them with water, dry

them with a Bunsen burner flame (*see* **Note 12**) and place them on a metal railing in order to keep the slide uncontaminated (especially the central part of the slides that will hold the fluidic chambers).

5. When the slide cools down, place two stripes of twin-sided adhesive tape parallel to the line defined by the two holes (**Fig. 3**). Keep about 6-10 mm distance between the two stripes and precisely place two additional stripes of tape on top of the first ones.

6. Carefully place a cover slip (Microscope cover slides 24x24 mm, Huber & Co. AG, Reinach, Switzerland) on the tapes, well centered on the slide covering the two holes. Using the top part of the tweezers apply some pressure at the corners to ensure that the cover slip sticks well to slide and is water tight.

7. Cut off the overhanging tape with a razor blade and quickly apply epoxy glue to seal the corners (**Fig. 3**, *see* **Note 13**). When the glue is dry (about 5-10 min) store each slide in a sterilized container (i.e. 50 mL falcon tubes, *see* **Note 14**).

3.3.3. Preparation of PGE-Coated Slides for Protein Experiments

In order to minimize nonspecific binding of proteins to the slide surface, polyethyleneglycol (PEG) is used as a passivating agent in single molecule experiments (**37,46**). The procedure below explains the preparation of PEG-coated quartz slides for single molecule experiments with Mss116 and D135-L14 RNA.

1. Clean the slides as described in the first four steps of Section 3.3.2.

2. After drying the slides with a flame, place the slides and coverslips into separate glass coplin jars, filled with 1M potassium hydroxide (KOH), and sonicate for ~1 hour.

3. Rinse the slides and coverslips first with distilled water then with methanol. Fill the jars with methanol and sonicate again for ~1 hour.

4. Clean a beaker with methanol for aminopropylsilation. Mix 1 mL of 3-

aminopropyltriethoxysilane reagent (Vectabond) kept in room temperature for ~ 1 hour with 100 mL methanol and 5 mL glacial acetic acid.

5. Remove methanol and fill the coplin jars containing the slides and coverslips with the previously prepared 3-aminopropyltriethoxysilane solution.

6. Incubate the slides and coverslips for 10 min and sonicate for 1 min. After sonication, the slides and coverslips should be incubated again for another 10 min.

7. Decant the aminopropylsilane mixture into the appropriate waste container and rinse the slides first with methanol and then with double distilled water.

8. Rinse the slides and coverslips again with methanol and dry them using nitrogen or argon.

9. Prepare the PEGylation buffer by dissolving 84 mg of sodium bicarbonate in 10 mL double distilled water and filter to sterilize.

10. Prepare PEGylation reaction solution (5 slides): Mix 4-8 mg of BIO-PEG-SCM, ~80 mg of m-PEG-SCM and 320 µL of the bicarbonate PEGylation buffer in 1 mL centrifuge vial. Vortex the solution to dissolve the PEG and then, centrifuge at 10'000 rpm for 1 min to remove bubbles.

11. In order to perform PEGylation, the slides should be placed in clean PEGylation reaction containers (use a clean pipette tip box and add water in the bottom of the container to maintain a humid environment).

12. Place 70 µL of PEGylation reaction solution onto the surface of each slide and slowly place the coverslips onto the slides covering the solution without creating any bubbles between the slide and the coverslip.

13. Close the containers and incubate overnight at room temperature in a dark place to allow the PEGylation reaction to occur.

14. Rinse slides and coverslips with ddH_2O and dry with nitrogen or argon as previously

explained.

15. Assemble the slides and coverslips following the steps 5 to 7 as explained in the section 3.3.2.

3.3.4. Beads Slide Preparation

The slides containing the bead solution do not need to have drilled holes. These slides can be completely sealed and re-used when necessary.

1. Rinse a cleaned slide (see procedure described in Section 3.3.2) with methanol and dry it under a flux of N_2. Place double layers of twin-sided adhesive tape parallel to each other and parallel to the long side of the slide about 6-10 mm apart (**Fig. 4a**). Place the cover slip (Microscope cover slips 24x24 mm) over the centre of the slide creating a microfluidic channel that will hold the beads solution. Cut off the excess of tape with a razor blade.

2. Load 50-70 μL of 0.5 M $MgCl_2$ into the chamber with a pipetman. Prepare 1/2500 diluted solution of FluoroSpheres® carboxylate-modified fluorescent beads in water and inject 50-70 μL of this solution into the chamber from the opposite side than used to load the $MgCl_2$ solution.

3. Check if it is possible to see the formed beads using the single molecule setup and whether the image quality and number of beads are satisfactory; see also Section 3.3.5). Seal the microfluidic chamber with epoxy glue (**Fig. 4b**, *see* **Note 15**).

3.3.5. smFRET Analysis of Sc.D135-L14 in 100 mM $MgCl_2$. Sample Preparation and Slide Loading.

The metal ion dependent folding of *Sc*.D135-L14 was studied using single molecule fluorescence experiments (**35**). Here, we explain the sample preparation and slide loading to monitor the folding of D135-L14 as an example case for such studies.

1. Prepare the pipette tips for loading: Cut off 2-3 mm of the cone end of the 200 μL pipette tip with a blade razor. Ensure that the tip tightly fits into the injection hole so that the solutions do not leak during loading but flush out only from the opposite hole (**Fig. 3**, *see* **Note 16**).

2. Prepare a fresh stock of oxygen scavenging solution by mixing ~50 μL (powder volume) of glucose oxidase, 12.5 μL catalase (*see* **Note 17**) and 100 μL of T50 buffer. This solution can be stored at 4°C, but used for no more than 3 days.

3. Prepare 10 mL of 1x Reaction Buffer by diluting 2 mL of 5x Reaction buffer in 8 mL of ddH_2O (autoclaved).

4. Prepare a 10% sugar/buffer solution by dissolving 100 mg D-glucose in 1 mL (total volume) of 1x Reaction buffer. Pass the solution through a 0.2 μm filter.

5. Prepare 10 μL stock solutions each of *Sc.*D135-L14 (2.5 μM), Cy3-DNA, Cy5-DNA and T-Biotin-DNA oligos (100 μM each). Keep the solutions on ice and the dye-DNA oligo solutions protected from light.

6. Prepare *solution A*: in a dark 200 μL eppendorf mix 2.5 μL of ddH_2O, 2 μL of 5x reaction buffer, 1 μL of β-mercaptoethanol (**1,47**) (*see* **Note 18**), 2 μL of *Sc.*D135-L14 stock solution and 0.5 μL of each Cy3-DNA, Cy5-DNA and T-Biotin-DNA stock solutions. Vortex, spin down, and heat to at 90°C for 45 seconds. Add 1 μL of 1 M $MgCl_2$ and incubate at 42°C for 15-20 min. Solution A now contains 10 μL of folded and labeled RNA at 0.5 μM D135-L14, 5 μM of each DNAs, 1 x Reaction buffer and 100 mM $MgCl_2$ concentration.

7. Start loading the slide: inject ~80 μL of biotinylated BSA solution and incubate for ~10 minutes to allow for uniform absorption to the slide surface (*see* **Notes 19 and 20**).

8. Meanwhile, prepare the oxygen scavenging system (OSS) by mixing 2 μL of the stock oxygen scavenging solution, 2 μL β-mercaptoethanol and 196 μL of the 10% sugar/buffer

solution. Incubate for 15-20 min before use to activate the scavenger system.

9. Wash the microfluidic chamber with ~200 μL of T50 buffer to remove the excess of BSA and inject ~200 μL of streptavidine solution. Incubate for ~10 min to optimize biotin binding.

10. In the meanwhile prepare *solution B*: Mix 1 μL *solution A* and 1 μL β-mercaptoethanol in 98 μL 1x reaction buffer.

11. Prepare *solution C* (the final sample solution to be injected): Mix 1 μL *solution B* and 2 μL β-mercaptoethanol in 197 μL 1x reaction buffer. The RNA is now ~25 pM.

12. Turn off the lights (*see* **Note 21**): Wash the microfluidic chamber with ~200 μL of 1x reaction buffer and inject ~200 μL *solution C*. Allow binding of the biotynilated RNA to the streptavidin for 7-10 min and inject ~200 μL OSS. Equilibrate for ~5 minutes before taking measurements.

3.3.6. Sample Preparation and Slide Loading for the Analysis of Sc.D135-L14 in the Presence of Mss116.

Protein-mediated folding of *Sc*.D135-L14 can be studied using single molecule fluorescence experiments with the DEAD-box protein Mss116 and ATP (**37**). This section explains the sample preparation and slide loading to monitor the folding of D135-L14 in the presence of Mss116 and ATP under near-physiological conditions.

1. Prepare *solution A* by mixing 2.7 μL of ddH_2O, 2 μL of 5x reaction buffer (400 mM MOPS pH 7.5 and 500 mM KCl), 1 μL of β-mercaptoethanol, 2 μL of *Sc*.D135-L14 stock solution and 0.5 μL of each Cy3-DNA, Cy5-DNA and T-Biotin-DNA stock solutions as explained in the section 3.3.5. Heat-anneal the sample for 45 seconds at 90°C and incubate at 30°C for 15-20 min after addition of 0.8 μL of 100 mM $MgCl_2$ to assure proper annealing and folding. At the end of this step we obtain a 10 μL solution containing 0.5 μM D135-L14,

5 μM of all DNA oligos, 100 mM KCl, 40 mM MOPS pH 7.5 and 8 mM $MgCl_2$.

2. Load the PEG-coated slide with ~80 μL of streptavidin solution and incubate for ~10 min to allow binding of streptavidin to the slide surface.

3. Meanwhile, prepare *solution B* and *solution C* as described above (step 9 in section 3.3.5.).

4. Wash the microfluidic chamber with ~200 μL of 1x Reaction buffer and inject ~200 μL of *solution C* (step 3). Incubate for 7-10 min for complete RNA binding.

5. Prepare the OSS with 25 nM Mss116 and 1-2 mM ATP by mixing 2 μL of stock oxygen scavenging solution and 2 μL of β-mercaptoethanol using 10% sugar-buffer solution for protein experiments (final volume 200 μL). Store this solution at 4°C until ready to inject.

6. Inject 200 μL of the oxygen scavenging system containing 25 nM Mss116 and 1-2 mM ATP (step 5) and incubate for 5 min.

3.3.7. Calibration of the Experimental Setup

In order to obtain the best possible single molecule data, first the instrument needs to calibrated using the fluorescent beads slide (see Section 3.3.4.). This allows to build a map that matches a molecule's signal in the donor channel with its corresponding signal in the acceptor channel.

1. Switch on the CCD camera and the acquisition software and cool the camera to -80°C.

2. Place a drop of ddH_2O on the high numerical aperture water immersion objective (60x) and place the fluorescent beads slide on the slide holder over the objective with the cover slip facing the objective (*see* **Note 22**). Secure the slide with stage clamps.

3. Position the prism onto the slide adding a drop of refraction index-matched immersion oil between slide and prism.

4. Switch the laser on and look for the fluorescent beads through the microscope's eye piece

using the appropriate filters. Focus the image (*see* **Note 23**) and centre the laser beam using the focusing lens placed in front of the prism. The set up is aligned when individual bright dots from isolated beads are visible (*see* **Note 24**).

5. To visualize the image onto the 512x512 pixel EM-CCD camera, switch to the side port. Focus and centre the signal in the observation window using the focusing lens until single beads are clearly visible as bright dots in the donor and acceptor channels (*see* **Note 25**). Search for at least 50 sharp and well focused beads, while avoiding large bright dots that may correspond to bead-aggregates.

6. Save ~30 frames to be further analyzed to generate the image map (*see* **Note 26** and Section 3.3.8).

3.3.7. Performing the Single Molecule FRET Experiment

After the instrument has been calibrated (Section 3.3.6.), proceed with the single molecule analysis of the RNA. The slides with the reaction solution is prepared as described (Section 3.3.5.). Always work in the dark when handling the fluorophore labeled RNA.

1. Five minutes after adding the oxygen scavenging system (step 12, Section 3.3.5) place the slide on the microscope's slide holder and visualize the single molecules as described above (steps 1-8).

2. An EM gain level of 400 is typically used to visualize the Cy3-Cy5 fluorophore pair (*see* **Note 27**).

3. Move the stage in order to observe a good set of single molecules and record a set of frames at the desired frame rate. Let the measurement run until photobleaching of the fluorophores is observed (photobleaching time depends on the laser intensity used).

4. Move the stage to another area of single molecules on the same slide to record a new set of frames. From a good slide, 8-10 movies can be collected yielding a large distribution of

single molecules for data analysis.

3.3.8. Data Analysis.

To obtain single molecule time trajectories, the location of single molecule peaks in the donor and acceptor channels is mapped using the image obtained from the immobilized fluorescent beads as a calibration map. The emission intensities of the donor and acceptor fluorophores for every recoded frame are obtained by integrating the corresponding peaks after background subtraction.

Since single molecule experiments can generate significantly large amounts of data, the analysis of single molecule FRET data requires the following specific criteria:

a. Anti-correlated donor and acceptor emission intensities.
b. Single-step photobleaching of fluorophores.
c. Stable emission intensities corresponding to single fluorophore.

Single molecule trajectories of the D135-L14 ribozymes provide valuable information about the folding of introns under different reaction conditions (**35,37**). Each single molecule FRET trajectory represents the folding behavior of an individual RNA molecule exhibiting different FRET states correspond to different structural conformations (**Fig. 5a**). In order to determine the distribution of different conformational states under given reaction conditions, FRET histograms can be constructed using FRET trajectories from more than 100 molecules, or until convergence (**Fig. 5b**). Therefore, FRET histograms represent the general effect of different reaction conditions on D135-L14 RNA folding. Under equilibrium conditions, the relative heights of peaks in the FRET histograms can be used to determine the relative stabilities of different conformational states.

In addition, FRET trajectories can be used to obtain valuable kinetic and mechanistic information about the D135-L14 RNA folding pathway using dwell times in each

conformational state. The dwell time is the amount of time a molecule spends in a given state before switching to the next state. Folding rate constants of D135-L14 can be determined by fitting the dwell-time distribution of each state with an exponential decay function (**Fig. 5c,d**). Alternatively, the complex single molecule trajectories can be analyzed using a Hidden Markov Model (HMM) for unbiased estimation of the number of distinct FRET states and the folding rate constants among those states (**37,48**). In addition, the order in which different conformational states appear in the trajectories also enables to distinguish between obligatory folding intermediates from off pathway intermediates.

The resulting folding rate constants for the D135-L14 ribozyme can then be used to calculate RNA folding free energy diagrams, which reveal the ribozyme's folding pathways (**37**).

3.3.9. Final Remarks

Single molecule FRET allows to study the folding and dynamics of even large RNAs and RNA protein complexes, at a so-far unprecedented resolution and provides information that is generally not accessible by bulk experiments. Monitoring the behavior of hundreds of single molecules in real time over several minutes reveals an astonishing diversity in behavior of these individual systems, e.g. short-lived intermediate folding states that are unknown from ensemble averaged experiments. From the individual time trajectories, rate constants of the single folding steps, binding events, possibly catalysis, as well as thermodynamic data can be calculated making this method a very powerful tool widely applicable in modern biophysical research.

Using the example of the Cy3/Cy5 labeled D135-L14 ribozyme derived from the yeast mitochondrial group II intron *Sc*.ai5γ group II intron, we here describe all steps starting from RNA transcription and isolation to the recording of single molecule time trajectories and data evaluation as well as the slight modifications applied when investigating RNA protein

interactions, e.g. in the Mss116-D135-L14 system. Every RNA behaves slightly differently, folds under different conditions and uses different cofactors for catalysis. In addition, the here described setup and methodology are used specifically in our groups and thus depend on each other. Generally, smFRET is not "black-box" technique, but requires hands-on optimization of the mostly self assembled setups as well as adjusting the methodology. Hence, every system has to be thoroughly optimized in order to get the most valuable data, but smFRET allows to readily implementing one's own ideas and directions thus contributing to this fast moving field of biophysical analysis.

4. Notes

1. The efficiency of energy transfer is given by $E_{FRET} = 1/[1+(R/R_0)^6]$, where R is the distance between the two fluorophores and R_0 the Förster radius at which 50% of energy transfer efficiency is observed:

$(R_0)^6 = 8.8 \times 10^{-28}\ \kappa^2\ n^{-4}\ Q_0 J$, $\quad J = \int f_D(\lambda)\,\varepsilon_a(\lambda)\,\lambda^4 \mathrm{d}\lambda$ where κ^2 is the factor describing the orientation of dipoles, n the refractive index of the medium, Q_0 the donor quantum yield in the absence of the acceptor, $f_D(\lambda)$ the fluorescence intensity of the donor, and $\varepsilon_a(\lambda)$ the molar extinction coefficient of the acceptor at the same wave length λ.

2. Confocal microscopy is mostly used for the detection of freely diffusing molecules in solution with low time resolutions because of the avalanche photodiode (ADP) and photomultipliers that are used in this technique (**34**). In contrast, TIRFM allows the excitation of smaller volumes, which limit the effect of noise background although the signal is detected with charge-couple device (CCD) cameras that work with higher time resolutions.

3. To investigate short RNA sequences (<60-80 nts) by FRET, the two fluorophores are covalently attached by direct insertion during chemical synthesis. In contrast, larger

constructs are obtained by *in vitro* transcription by T7 RNA polymerase, which prevents the insertion of modified nucleotides in the middle of a sequence.

4. The underlying construct *Sc.*D135 containing only D1, D3 and D5 is the best characterized group II intron construct that contains all necessary units for folding and catalytic activity (**38**). Control experiments have shown that also *Sc.*D135-L14 retains equivalent catalytic activity (**35**).

5. θ_c depends on the indexes of refraction of the quartz slide and buffer solution. The right incidence angle is controlled by regulating the height and distance of the mirror M, as described in detailed in ref (**33**).

6. This setup is known as prism-based TIRF. Alternatively in the objective-based TIRF method, the evanescent wave can be generated through the objective (**33**). Here, the alignment of the laser beam is more complicate and this strategy is used only when the presence of the prism over the slide is an impediment for the type of experiment to perform.

7. Extinction coefficients of the nucleotides (M^{-1} cm^{-1}) are: ε_{ATP} (260 nm) = $15.4 \cdot 10^3$, ε_{UTP} (260 nm) = $10.0 \cdot 10^3$, ε_{CTP} (270 nm) = $9.0 \cdot 10^3$, ε_{GTP} (249 nm) = $13.7 \cdot 10^3$.

8. The extinction coefficients ε can be estimated using web-based tools, e.g. http://biophysics.idtdna.com/UVSpectrum.html or http://www.owczarzy.net/abstr11.htm. These calculation tools are based on the "nearest neighbor" method meaning that they take into account not only the extinction of each nucleobase, but also the identity of the neighboring bases (**49,50**).

9. Approximate fluorescence excitation/emission of these beads is 580/606 nm. Alternatively, crimson fluorescent FluoroSpheres® carboxylate-modified microspheres (625/645 nm), 0.2 μm, 2% in distilled water, 2mM azide, from Invitrogen, can be used.

10. The concentration of homemade T7 is usually not determined. We concentrate T7 as much as possible (**45**). Hence, the optimal amount needs to be optimized in transcription

trials on an analytical scale (50 μL transcription). Transcription efficiency strongly varies depending on the RNA sequence, plasmid concentration, T7 batch, $MgCl_2$ and NTP concentration (see also Chapter 16, Section 3.1.).

11. An image of such a native gel experiment is reported in Fig. S2 of the Supporting Information of ref (**35**).

12. The slides will break easily if held for too long over the flame. Move the slide with the tweezers towards the flame and slowly move it back. Repeat this for about 10 seconds until the slide is dry.

13. Use just the minimal amount of glue to seal the open corners, since excess epoxy glue penetrates into the microfluidic chamber by capillary forces and plugs the holes.

14. Slides can be stored for about 6-8 weeks. Used slides can be recuperated: Soften the glue by boiling the slides in dd H_2O for 20 min or until the glue turns yellowish. Use a razor blade to remove the cover slip and the glue and proceed with the cleaning as described in the beginning of this Section 3.2.2.

15. Calibration of the instrument can be performed with the same beads slide for many weeks as the beads solution in $MgCl_2$ is photostable.

16. The same tip can be used for all the injections described below.

17. Collect the supernatant of the suspension. Alternatively aliquot 16 μL of suspension in an eppendorf tube, centrifuge for 30 sec and take 12.5 μL of the supernatant.

18. β-mercaptoethanol quenches the triplet state of the dyes, which helps to achieve a steady light emission minimizing any blinking effects (**1,47**).

19. Ideally, both sample preparation and injection into the slide should be carried out in the room set up for single molecule experiments. Keep shut the black curtains of the room during the sample injection, and work close to the microscope thus the slide can be easily transferred for analysis after the injection.

20. Each solution is slowly and completely injected, carefully avoiding formation of bubbles and allowing the excess of solution to flow out of the opposite hole. After injection remove the tip from the injection hole by keeping down the plunger button of the pipetman to avoid sucking out the solution again.

21. The injection of *solution C* must occur in a dark room assisted only by low power LED lights. We use USB notebook led lights (5V, 48mA) plugged using a 2-port USB charger.

22. Make sure that the slide is resting on the holder and not on the lens of the objective.

23. Take care not to jam the objective into the slide, cracking the slide or, even worse, scratching the lens of the objective.

24. If the image cannot be found, it is recommended to remove the filter and use a low-magnification objective (e.g. 10x) to pre-align the laser beam on the image centre. Then switch back to the high numerical aperture objective. Make sure that still a drop of water is on the lens.

25. Usually no or only a very low EM gain level (~20) is required to visualize the fluorescent beads.

26. We use the "Run Till Abort" acquisition mode of the Labview program that allows the saving of each picture frame of the movie recorded by the CCD camera at the respective frame rate. This movie containing the sequence of frames is saved to the hard drive as one large *.pma file, which is just a binary file containing all saved frames. This file is then processed with IDL (ITT VIS) scripts to extract the single molecules time trajectories.

27. It is crucial to turn off **all** lights when the EM gain function is enabled, to avoid fast aging of the CCD camera.

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Figure Legends

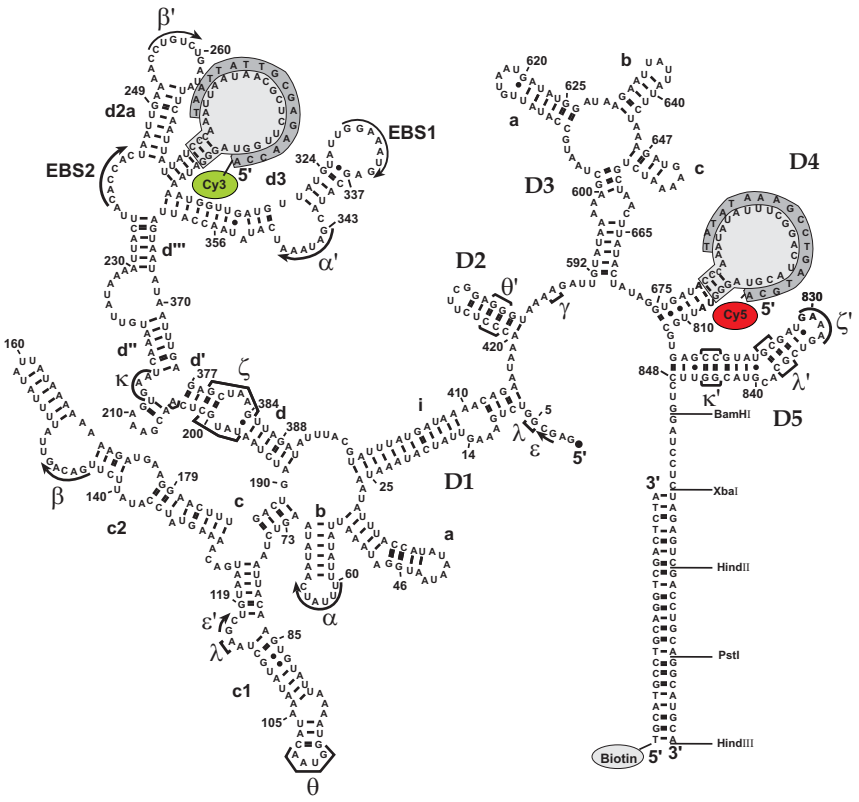
Figure 1. Secondary structure of the *Sc.*D135-L14 ribozyme originating from the wild-type *Sc.*ai5γ group II intron from *S. cerevisiae*. In *Sc.*D135-L14 D2 is reduced to a hairpin, D6 is deleted, and two modular loops (L1 and L4) are inserted to permit annealing of DNA-Cy3 and DNA-Cy5 (highlighted in dark). The 3'-end of the intron is elongated with a sequence suitable for the annealing of a biotinylated DNA (also in dark) in order to immobilize the construct on a streptavidin coated slide. The numbering corresponds to the wild type *Sc.*ai5γ and tertiary contacts (Greek letters) as well as exon/intron binding sequences (EBS and IBS) are indicated.

Figure 2. smFRET TIRF setup: **a)** The Cy3/Cy5-Labelled and biotinylated *Sc.*D135-L14 ribozyme is loaded into the microfluidic chamber of a self made slide coated with streptavidine. **b)** The mirror M and the lens L focus the excitation laser beam to the slide with an angle larger than θ_C to achieve total internal reflection. The resulting evanescent wave excites the molecules within 100-150 nm from the slide surface. The emitted light of the donor D and acceptor A is collected through the objective into the light-tight box where the signals from D and A are separated and detected by the CCD camera. **c)** Single molecules detected through donor and acceptor emission are visible as bright dots in two parallel images. **d)** Data analysis yields FRET a time trajectory of each single molecule (the shown time trace of *Sc.*D135-L14 at 100 mM $MgCl_2$ adapted from ref (**35**)).

Figure 3. Schematic representation of slide preparation with the microfluidic channel, into which the sample is loaded (see also text in Sections 3.3.2. and 3.3.3.).

Figure 4. Preparation of beads slides. The two sets of double sticky tape are placed parallel to the slide and covered with a cover slip. The solutions are filled from the opposite ends (see also text in Sections 3.3.4.).

Figure 5. Single molecule data analysis. **a)** A typical FRET trajectory showing the behavior of a single D135-L14 ribozyme in the presence of Mss116 and ATP under near-physiological conditions (8 mM $MgCl_2$, 100 mM KCl and 40 mM MOPS pH 7.5). **b)** FRET histogram showing the distribution of three structural conformations: the extended intermediate state (I), the folded intermediate state (F) and the native state (N). **c)** D135-L14 minimal folding pathway showing folding rate constants k_1, k_{-1}, k_2 and k_{-2}. **d)** A typical dwell time distribution fit to a single exponential decay to obtain a rate constant for a given transition. Figures are adapted from references (**35,37**).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D4
D5
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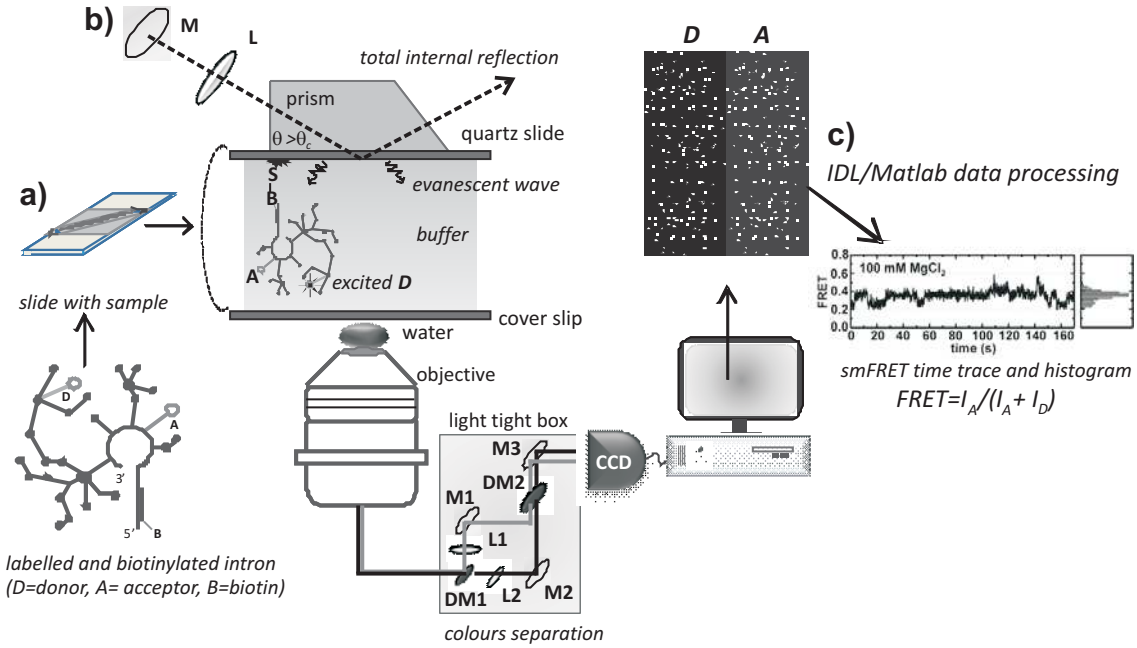
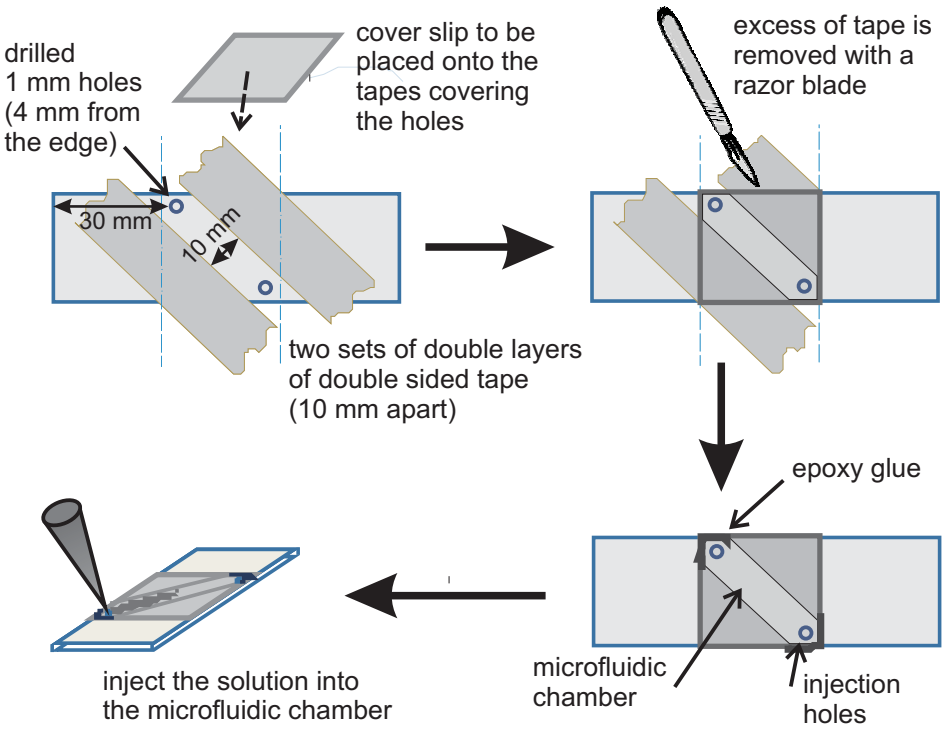
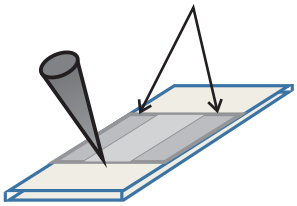
c
Cy3
Cy5
5'
3'
BamHI
XbaI
HindII
PstI
HindIII
Biotin


b)
M
L
total internal reflection
prism
θ >θc
quartz slide
S
B
evanescent wave
buffer
A
excited D
a)
slide with sample
water
cover slip
objective
light tight box
M3
DM2
M1
L1
DM1
L2
M2
CCD
D
A
c)
IDL/Matlab data processing
100 mM MgCl2
FRET
time (s)
smFRET time trace and histogram
FRET=IA/(IA+ ID)
labelled and biotinylated intron
(D=donor, A= acceptor, B=biotin)
colours separation

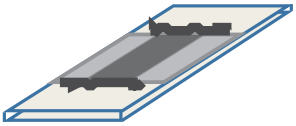

drilled
1 mm holes
(4 mm from
the edge)
cover slip to be
placed onto the
tapes covering
the holes
30 mm
10 mm
two sets of double layers
of double sided tape
(10 mm apart)
excess of tape is
removed with a
razor blade
epoxy glue
microfluidic
chamber
injection
holes
inject the solution into
the microfluidic chamber

a) double layers of double sided tape with cover slip on top

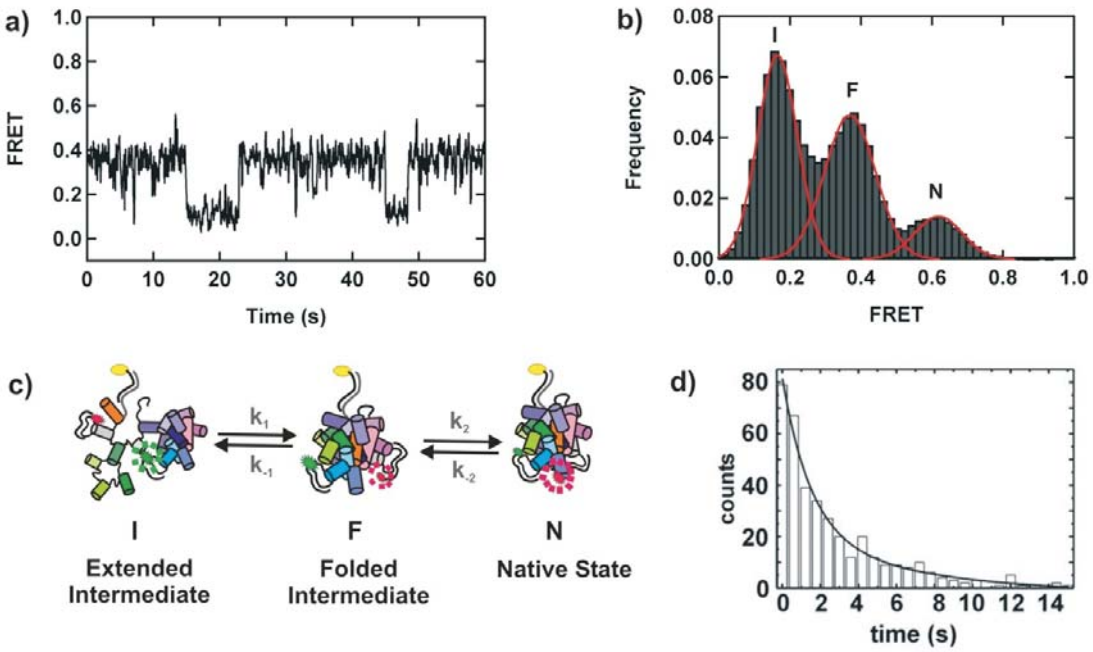


inject the solution into the microfluidic chamber

b)



beads slide sealed with epoxy glue


a)
FRET
Time (s)
b)
Frequency
FRET
I
F
N
c)
k1
k-1
k2
k-2
I
Extended
Intermediate
F
Folded
Intermediate
N
Native State
d)
counts
time (s)